

# Genetic similarity of natural populations and plantations of *Pinus roxburghii* Sarg. in Nepal

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## Abstract

• Genetic structures of five population pairs each consisting of one natural population and one neighbouring plantation of *Pinus roxburghii* Sarg. in Nepal were assessed using nuclear (nSSRs) and chloroplast microsatellite loci (cpSSRs).  
• The mean number of alleles at nSSRs loci in natural populations was 5.0 compared to 4.93 in plantations while the average observed heterozygosities were the same in both groups ( $H_0 = 0.50$ ). Most of the alleles were common to all the populations, indicating that the populations correspond to a single genetic entity. Similarly forty-seven haplotypes were observed in natural populations compared to fifty haplotypes in plantations. Mean haplotype diversities of natural populations (0.953) and plantations (0.955) were very similar. Genetic diversity of *Pinus roxburghii* was relatively high with low or no evidence of inbreeding while genetic differentiation among all populations was very low (about 1%).  
• The very low differentiation among natural populations indicates efficient long- distance gene flow among populations resulting in homogeneous genetic structures at least at selectively neutral loci. Even though the harvest and production of seeds and seedlings was largely uncontrolled, genetic structures of most plantations show no signs of reduced variation, inbreeding or other negative effects compromising the adaptedness or adaptability of planted forests.

## Mots-clés :

diversité génétique /  
différenciation génétique /  
matériels forestiers reproducteurs /  
microsatellites nucléaires /  
microsatellites chloroplastiques

## Résumé – Similitude génétique des populations naturelles et des plantations de *Pinus roxburghii* Sarg. au Népal.

• Les structures génétique de cinq paires de populations composées chacune d'une population naturelle et d'une plantation voisine de *Pinus roxburghii* Sarg. au Népal ont été évalués à l'aide de loci nucléaires (nSSRs) et de loci microsatellites chloroplastiques (cpSSRs).  
• Le nombre moyen d'allèles à loci nSSRs dans les populations naturelles était de 5,0 comparé à 4,93 dans les plantations alors que la moyenne des hétérozygoties observées étaient les mêmes dans les deux groupes ( $H_0 = 0,50$ ). La plupart des allèles étaient communs à toutes les populations, indiquant que les populations correspondent à une seule entité génétique. De même, quarante-sept haplotypes ont été observés dans les populations naturelles par rapport à cinquante haplotypes dans les plantations. La diversité moyenne des haplotypes des populations naturelles (0,953) et des plantations (0,955) étaient très similaires. La diversité génétique de *Pinus roxburghii* a été relativement importante, avec peu ou pas de preuve de consanguinité tandis que la différenciation génétique entre les populations était très faible (environ 1 %).  
• Le très faible différenciation entre les populations naturelles indique des flux de gènes efficaces à longue distance entre les populations issues de structures génétiques homogènes avec au moins une sélectivité neutre des loci. Même si la récolte et la production de semences et de plants ont été largement incontrôlées, les structures génétiques de la plupart des plantations ne montrent aucun signe de réduction de la variation de la consanguinité ou d'autres effets négatifs compromettant la faculté d'adaptation des forêts plantées.

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## 1. INTRODUCTION

An understanding of the degree of diversity within and between populations of a species is required to support continuously rising plantation programs and to provide information for the conservation of the species' genetic resources since geographically separated populations are expected to have different genetic compositions (e.g. Sharma et al., 2002). Genetic diversity is of prime importance for species persistence (Wang and Szmidt, 2001), since the evolutionary adaptive potential of populations depends on genetic variation patterns (Finkeldey and Hattermer, 2007; Siregar, 2000). Within each tree species, the amount and distribution of genetic variation determine its evolutionary adaptability. Consequently, genetic variation is crucial for the long-term stability of forest ecosystems (Sharma et al., 2002).

Genetic structures of numerous plant populations have been altered by humans for many generations (Finkeldey and Hattermer, 2007). This applies in particular to tree species used for the establishment of plantations. Genetic structures of plantations are often assumed to strongly differ from natural stands, to exhibit reduced levels of genetic variation and, accordingly, reduced adaptive potentials in comparison to natural stands (e.g. Pandey et al., 2004). Reduced adaptability is particularly likely if seeds are harvested and seedlings are raised without appropriate control and in the absence of efficient legal regulations governing the marketing of forest reproductive material (Nason, 2001). Legislation on forest reproductive material is currently either not existing or poorly enforced in most developing countries. Thus, many routinely established plantations of tropical forest tree species are assumed to show low levels of genetic diversity, i.e. to exhibit a 'narrow genetic base', since reproductive material was harvested and raised uncontrolled. However, few studies examine the impact of the harvest of forest reproductive material and the raising of seedlings in nurseries on the genetic structures of the resulting plantations in subtropical and tropical regions.

*Pinus roxburghii* Sarg. is a wind-pollinated, monoecious subtropical pine species. It is found in a fairly continuous distribution throughout the monsoon belt between 72° E and 95° E longitude and 27° N and 35° N latitude; and is abundant on the hill slopes of both the western and eastern Himalayas between altitudes of 450 m to 2 300 m above sea level (Arya et al., 2000). Naturally, it is distributed from Bhutan (only in drier areas), Northern India (Jammu and Kashmir, Punjab, Himanchal Pradesh, Uttarakhand, Sikkim), Nepal, south of Tibet and Pakistan to Afghanistan (Dogra, 1985; Yi and Raven, 1999).

*P. roxburghii* has outnumbered all other species in afforestation programs in its natural zone of occurrence. It is by far the most widely planted forest tree species both in government managed forests and community forests in Nepal. It comprises 17% of the total forest area of Nepal. It is also a species widely planted by individual farmers because of its high survival rate and ease of plantation establishment and management (Jackson, 1994). However, the origin of plant material for plantations is usually unknown.

In this study, the genetic structure of *P. roxburghii* was assessed using nuclear and chloroplast microsatellite markers. The central objective of the study was to assess the genetic variation within natural populations of *P. roxburghii* in Nepal and to compare results with variation patterns in plantations. Four hypotheses were formulated: (i) the different origin of natural and planted populations is reflected by different genetic structures; (ii) plantations show lower levels of genetic variation than natural populations due to bottlenecks during the harvesting of reproductive material and raising of seedlings; (iii) genetic differentiation between plantations and neighboring natural populations is high; and (iv) genetic variation is spatially structured in natural populations, i.e. distantly located natural populations are genetically more differentiated from each other than neighboring populations, but not in plantations.

## 2. MATERIALS AND METHODS

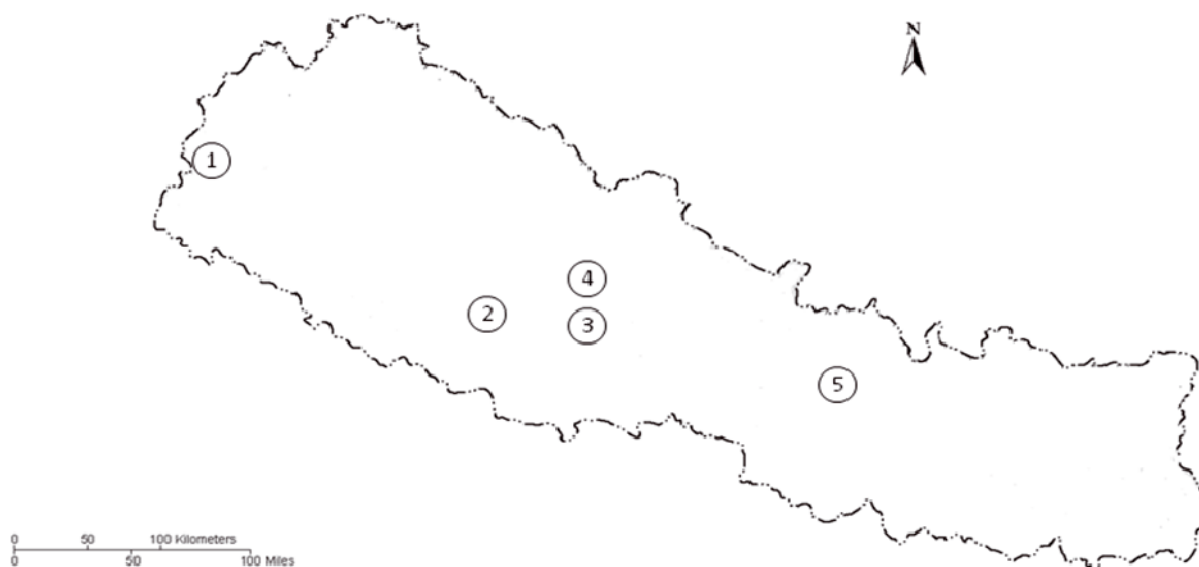
### 2.1. Plant material and DNA extraction

Five pairs of natural (NR) and planted (PL) populations were selected from four different development regions throughout the natural distribution range of *P. roxburghii* in Nepal in order to have a population sample that is representative for the whole country both for natural forests and plantations. Population pairs were selected on the basis of pair availability. Both populations from the same pair were located in similar altitudes and experienced similar climatic conditions. The distance between two populations from the same pair varied from 1 km to 5 km. Populations were sampled in (1) Dadeldhura (1 745 m asl), (2) Salyan (1 457 m asl), (3) Palpa (1 350 m asl), (4) Parbat (946 m asl), and (5) Bhaktapur (2 163 m asl) (Fig. 1).

Forty trees from each population were sampled. The first tree was selected randomly and the next at the distance of double tree height of the previous sampled tree. Twigs with terminal buds were collected from individual trees and were packed separately in small labelled plastic bags. DNA was extracted from needles using the DNeasy 96 Plant Kit (Qiagen). DNA extraction followed the protocol of the supplier.

### 2.2. PCR amplification for nuclear microsatellites markers

Eight nuclear microsatellites (nSSRs) PtTX2123, PtTX3020, PtTX3025, PtTX3118, PtTX3030, PtTX3107 (Auckland et al., 2002), LOP1 and LOP3 (Liewlaksaneeyanawin et al., 2004) were selected for the study. Polymerase Chain Reaction (PCR) for PtTX2123, PtTX3020, PtTX3025, PtTX3118, PtTX3030 and PtTX3107 was performed in a 15 µL volume containing about 20 ng DNA, 1 × Qiagen PCR buffer, 2.5 mM of MgCl<sub>2</sub>, 0.5 mM of each dNTPs, 0.67 µM of each forward and reverse primers, 0.5 × Q solution (Qiagen) and 1 U of Taq polymerase (Qiagen). Each forward primer was labelled with blue (6-FAM) or green (HEX) fluorescent dyes. PCR for LOP1 and LOP3 was performed in a 10 µL volume containing 20 ng DNA, 1 × Qiagen PCR buffer, 1.5 mM of MgCl<sub>2</sub>, 0.5 mM of each dNTP, 0.67 µM of each forward and reverse primers, 0.67 × Q solution



**Figure 1.** Map of Nepal showing locations of investigated population pairs. (1): Dadeldhura natural population and Dadeldhura plantation, (2): Salyan natural population and Salyan plantation, (3): Palpa natural population and Palpa plantation, (4): Parbat natural population and Parbat plantation, (5): Bhaktapur natural population and Bhaktapur plantation.

(Qiagen) and 1 U of Taq polymerase (Qiagen). The PCR amplification was carried out in a Peltier Thermal Cycler PTC -200 (MJ Research). The PCR profile consisted of 15 min denaturation at 95 °C with 5 cycles of denaturation at 94 °C for 1.3 min, 55 °C annealing for 1.3 min and 72 °C extension for 1.3 min, decreasing 1 °C per cycle followed by 33 cycles of denaturation at 94 °C for 1.3 min, 50 °C annealing for 1.3 min and 72 °C extension for 1.3 min followed by the final extension of 72 °C for 10 min. For LOP 1 and 3, the PCR profile consisted of 15 min denaturation at 95 °C, followed by 30 cycles of denaturation at 94 °C for 1 min, 60 °C annealing for 1 min and 1 min extension at 72 °C, with final extension at 72 °C for 8 min.

### 2.3. PCR amplification for chloroplast microsatellite markers

The four chloroplast microsatellite loci Pt71936, Pt63718, Pt87268 and Pt15169 (Vendramin et al., 1996) were used for the study. PCR amplification was performed in a 15 µL volume containing 1 × Qiagen PCR buffer, 2.5 mM of MgCl<sub>2</sub>, 0.5 mM of each dNTP, 0.67 µM of each forward and reverse primer, 1U of Taq polymerase (Qiagen) and about 20 ng DNA. Each forward primer was labelled with blue (6-FAM) or green (HEX) fluorescent dyes. Amplification was carried out in a Peltier Thermal Cycler PTC -200 (MJ Research). PCR amplification followed the method described by Vendramin et al. (1996) with slight modifications. The PCR profile consisted of 15 min denaturation at 95 °C, 8 min enzyme addition at 80 °C, followed by 25 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min and 1 min extension at 72 °C, with a final extension step at 72 °C for 8 min.

### 2.4. Fragment analysis

Amplified PCR products were diluted according to their intensity after agarose gel electrophoresis and were then run on the

ABI PRISM Genetic Analyser 3100, with the internal size standard GS 500 ROX (Applied Biosystems). Data were analysed using Genescan 3.7 and Genotyper 3.7 softwares (Applied Biosystems).

## 2.5. Data Analysis

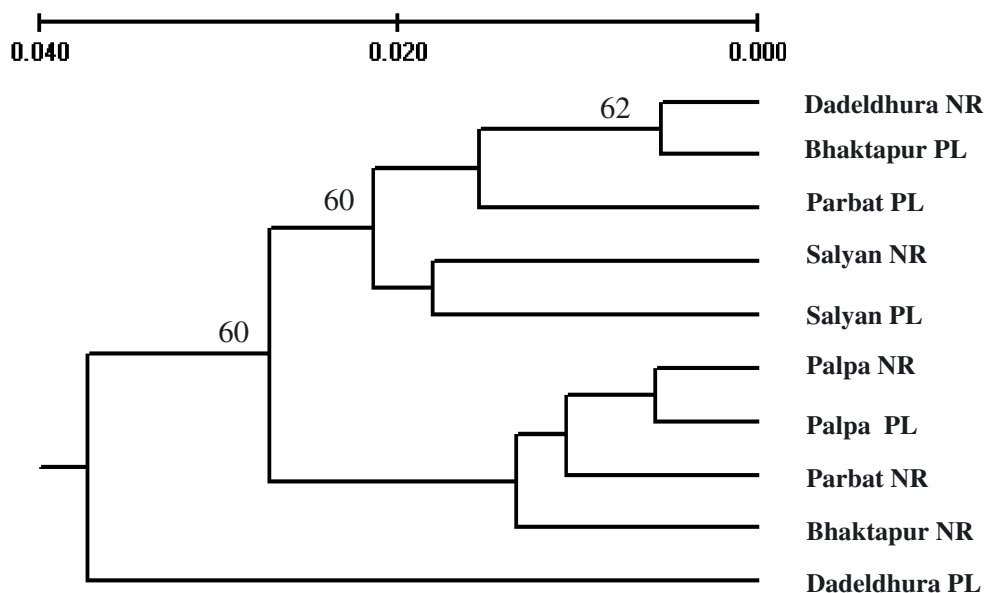
### 2.5.1. Analysis of nuclear microsatellites

For the analysis of nSSR data, allelic and genotypic frequencies, effective numbers of alleles ( $N_e$ ; Nei, 1975), observed and expected heterozygosity ( $H_o$  and  $H_e$  respectively; Nei, 1975) and fixation index ( $F_i$ ; Wright, 1965) were calculated using GenAlEx6 (Peakall and Smouse, 2005). Overall  $F_{ST}$  (Weir and Cockerham, 1984) was estimated using the software FSTAT version 2.9.3 (Goudet, 2001). Because of its dependence on the level of genetic variation a standardized genetic differentiation measure was calculated according to Hedrick (2005). Pairwise  $F_{ST}$  values were calculated according to Wright (1965) using the software Arlequin (Excoffier et al., 2005).

A non-hierarchical analysis of molecular variance (AMOVA; Excoffier et al., 1992) was applied to estimate within and among population differentiation for natural populations and plantations and between population types (natural and planted). Significance tests were performed by 1 023 permutations.

Genetic distance matrices and UPGMA dendrograms were computed based on Nei's genetic distance (Nei, 1972) using the software POPULATIONS 2.0 (Langella, 2002). Statistical support was assessed by means of 1 000 bootstrap replicates over loci.

Mantel tests (Mantel, 1967) were performed using the software NTSYS-pc version 2.0 (Rohlf, 1998) and GenAlEx6 (Peakall and Smouse, 2005) in order to test for a significant correlation between the matrix of geographic and genetic distances within the group of natural populations and plantations, respectively.



**Figure 2.** UPGMA dendrogram based on Nei (1972) minimum genetic distance between all studied natural populations and plantations (nSSRs). Bootstrap values based on 10000 permutations (only bootstrap values above 50) are indicated in each node.

### 2.5.2. Analysis of chloroplast microsatellites

The multilocus genotypes observed at the two polymorphic cpSSRs were jointly interpreted as haplotypes. Chloroplast haplotype variation within populations was calculated by estimating the total number of haplotypes  $N$  by direct count, the effective number of haplotypes ( $Ne$ ; Nei, 1978), haplotype diversity as the probability that two haplotypes chosen at random from a population are different ( $h \leq 1$ ; Nei, 1978) and genetic distance ( $D$ ; Nei, 1978) using GenALEx6 (Peakall, and Smouse, 2005) software.

For the clustering analysis, Nei's standard genetic distance (Nei, 1978) was calculated for each pair of populations using AFLP-SURV (Vekemans, 2002) and the dendrogram was constructed with the programs NEIGHBOUR (UPGMA algorithm) and CONSENSE (expanded majority rule approach) of the package PHYLIP (Felsenstein, 1989) release 3.66. Statistical support of the clusters was assessed by means of 1 000 bootstrap replicates over loci. Analysis of molecular variance and Mantel tests were conducted as described for nSSRs.

## 3. RESULTS

### 3.1. Nuclear microsatellites

Out of eight nSSR loci tested in 16 samples from all populations, seven amplified, and one (PtTX3025) failed to amplify. Among these seven loci, four (PtTX3020, PtTX3118, PtTX3030 and LOP3) were monomorphic and three (PtTX2123, PtTX3107 and LOP1) revealed polymorphisms. The results are based on the three polymorphic loci.

### 3.2. Genetic variation within populations

At the three analysed loci, 23 alleles were detected with an average of 7.66 alleles per locus in natural populations.

Slightly lower variation, i.e. 21 alleles with an average of 7 alleles per locus, was observed in plantations. A summary of the genetic variation parameters for each of the three nuclear microsatellites and for each of the populations as well as mean values over all loci are given in Table 1. The mean effective number of alleles in natural populations ranged from 2.21 (Parbat) to 2.74 (Salyan), in plantations it ranged from 2.24 (Parbat) to 2.85 (Salyan). In natural populations, observed heterozygosity ( $H_o$ ) for all loci ranged from 0.45 (Palpa) to 0.55 (Salyan) with a mean value of 0.50. Within plantations,  $H_o$  ranged from 0.42 (Palpa) to 0.54 (Dadeldhura) with a mean value of 0.50 as well. In natural populations, expected heterozygosity ( $H_e$ ) for nSSRs loci varied from 0.50 (Parbat and Dadeldhura) to 0.56 (Salyan) with a mean value of 0.52. In plantations  $H_e$  ranged from 0.49 (Palpa) to 0.56 (Bhaktapur) with the same mean value as in natural populations. The mean values of the fixation indices for three populations (Bhaktapur NR, Dadeldhura PL and Parbat PL) were slightly negative. For most of the populations fixation indices were close to zero. Only the natural and planted population from Palpa showed high and positive fixation indices of 0.17 and 0.18, respectively.

### 3.3. Genetic differentiation among populations

Genetic distances ranged from 0.005 between Dadeldhura NR and Bhaktapur PL to 0.048 between Dadeldhura PL and Parbat PL. All genetic distances between populations were low with an average of 0.026. The UPGMA dendrogram based on Nei's (1972) genetic distance (Fig. 2) showed there was no clear grouping of population pairs (natural/ planted) from one region. For most clades there was only low (non-significant) bootstrap support. Clusters with significant bootstrap support

**Table I.** Summary of genetic variation parameters at three nuclear microsatellites for each natural and planted population.

| Natural    |           | Dadeldhura | Salyan | Palpa | Parbat | Bhaktapur | Mean over NR |
|------------|-----------|------------|--------|-------|--------|-----------|--------------|
| LOP1       | <i>N</i>  | 40         | 39     | 40    | 40     | 40        | 39.8         |
|            | <i>Na</i> | 4          | 5      | 5     | 5      | 5         | 4.8          |
|            | <i>Ne</i> | 1.26       | 1.46   | 1.66  | 1.41   | 1.62      | 1.48         |
|            | <i>Ho</i> | 0.23       | 0.28   | 0.33  | 0.33   | 0.38      | 0.31         |
|            | <i>He</i> | 0.21       | 0.32   | 0.4   | 0.29   | 0.39      | 0.32         |
|            | <i>F</i>  | −0.09      | 0.1    | 0.18  | −0.12  | 0.02      | 0.02         |
| PtTX2123   | <i>N</i>  | 40         | 37     | 40    | 40     | 35        | 38.4         |
|            | <i>Na</i> | 8          | 7      | 5     | 5      | 5         | 6            |
|            | <i>Ne</i> | 4.5        | 4.33   | 3.62  | 3.09   | 3.67      | 3.84         |
|            | <i>Ho</i> | 0.75       | 0.81   | 0.65  | 0.65   | 0.83      | 0.74         |
|            | <i>He</i> | 0.79       | 0.78   | 0.73  | 0.68   | 0.74      | 0.74         |
|            | <i>F</i>  | 0.04       | −0.05  | 0.1   | 0.04   | −0.14     | 0            |
| PtTX3107   | <i>N</i>  | 40         | 40     | 40    | 40     | 40        | 40           |
|            | <i>Na</i> | 3          | 5      | 3     | 5      | 5         | 4.2          |
|            | <i>Ne</i> | 1.96       | 2.44   | 1.96  | 2.12   | 1.86      | 2.07         |
|            | <i>Ho</i> | 0.48       | 0.55   | 0.38  | 0.45   | 0.43      | 0.46         |
|            | <i>He</i> | 0.5        | 0.6    | 0.49  | 0.54   | 0.47      | 0.52         |
|            | <i>F</i>  | 0.03       | 0.07   | 0.23  | 0.15   | 0.08      | 0.11         |
| Mean       | <i>N</i>  | 40         | 38.67  | 40    | 40     | 38.33     | 39.4         |
|            | <i>Na</i> | 5          | 5.67   | 4.33  | 5      | 5         | 5            |
|            | <i>Ne</i> | 2.58       | 2.74   | 2.41  | 2.21   | 2.38      | 2.46         |
|            | <i>Ho</i> | 0.48       | 0.55   | 0.45  | 0.48   | 0.54      | 0.5          |
|            | <i>He</i> | 0.5        | 0.56   | 0.54  | 0.5    | 0.53      | 0.53         |
|            | <i>F</i>  | 0.01       | 0.04   | 0.17  | 0.02   | −0.01     | 0.04         |
| Plantation |           | Dadeldhura | Salyan | Palpa | Parbat | Bhaktapur | Mean over PL |
| LOP1       | <i>N</i>  | 39         | 38     | 40    | 40     | 40        | 39.4         |
|            | <i>Na</i> | 4          | 5      | 5     | 4      | 6         | 4.8          |
|            | <i>Ne</i> | 1.27       | 1.32   | 1.44  | 1.53   | 1.58      | 1.43         |
|            | <i>Ho</i> | 0.23       | 0.26   | 0.23  | 0.35   | 0.38      | 0.29         |
|            | <i>He</i> | 0.22       | 0.24   | 0.31  | 0.35   | 0.37      | 0.3          |
|            | <i>F</i>  | −0.09      | −0.09  | 0.26  | −0.01  | −0.01     | 0.01         |
| PtTX2123   | <i>N</i>  | 38         | 36     | 39    | 37     | 40        | 38           |
|            | <i>Na</i> | 6          | 6      | 5     | 5      | 6         | 5.6          |
|            | <i>Ne</i> | 3.29       | 4.86   | 3.5   | 4.38   | 4.1       | 4.03         |
|            | <i>Ho</i> | 0.76       | 0.83   | 0.74  | 0.76   | 0.68      | 0.75         |
|            | <i>He</i> | 0.7        | 0.81   | 0.72  | 0.78   | 0.77      | 0.76         |
|            | <i>F</i>  | −0.1       | −0.05  | −0.04 | 0.02   | 0.11      | −0.01        |
| PtTX3107   | <i>N</i>  | 37         | 39     | 40    | 39     | 40        | 39           |
|            | <i>Na</i> | 5          | 4      | 3     | 5      | 5         | 4.4          |
|            | <i>Ne</i> | 2.38       | 2.37   | 1.78  | 1.66   | 2.19      | 2.08         |
|            | <i>Ho</i> | 0.62       | 0.44   | 0.3   | 0.41   | 0.5       | 0.45         |
|            | <i>He</i> | 0.59       | 0.59   | 0.45  | 0.4    | 0.55      | 0.51         |
|            | <i>F</i>  | −0.07      | 0.25   | 0.32  | −0.03  | 0.08      | 0.11         |
| Mean       | <i>N</i>  | 38         | 37.67  | 39.67 | 38.67  | 40        | 38.8         |
|            | <i>Na</i> | 5          | 5      | 4.33  | 4.67   | 5.67      | 4.93         |
|            | <i>Ne</i> | 2.31       | 2.85   | 2.24  | 2.52   | 2.62      | 2.51         |
|            | <i>Ho</i> | 0.54       | 0.51   | 0.42  | 0.51   | 0.52      | 0.5          |
|            | <i>He</i> | 0.5        | 0.54   | 0.49  | 0.51   | 0.56      | 0.52         |
|            | <i>F</i>  | −0.08      | 0.03   | 0.18  | −0.01  | 0.05      | 0.04         |

*N*: sample size; *Na*: number of alleles; *Ne*: effective number of alleles; *Ho*: observed heterozygosity; *He*: expected heterozygosity; *F*: fixation index.



**Table II.** Summary of the non-hierarchical analysis of molecular variance (AMOVA) for natural populations and plantations at nSSRs.

| Source of variation            | <i>df</i> | Sum of squares | Variation component | variation | <i>p</i> value |
|--------------------------------|-----------|----------------|---------------------|-----------|----------------|
| Among groups                   | 1         | 1.029          | 0.000               | 0%        | 0.66569        |
| Among population within groups | 8         | 10.838         | 0.008               | 1%        | < 0.05         |
| Within population              | 788       | 591.046        | 0.750               | 99%       | < 0.001        |

*df*: degree of freedom; *p* value: the probability of obtaining a more extreme component estimate by chance.

(> 50) are marked in Figure 2. Considering the five studied natural populations, the Mantel test revealed no significant correlation of genetic and geographic distances for both natural populations ( $r = 0.1814$ ,  $P = 0.6869$ ) and plantations ( $r = 0.2105$ ,  $P = 0.340$ ).

The proportion of the total genetic variation distributed among populations computed using  $F_{ST}$  was 0.6% (standardized  $F_{ST} = 1.95\%$ ) for natural populations and 1.4% (standardized  $F_{ST} = 4.43\%$ ) for plantations. AMOVA revealed that only 1.0% of the total genetic variation occurred among populations in both populations types. AMOVA among the two groups (natural populations and plantations) revealed virtually no differentiation between the groups (Tab. II). Pairwise  $F_{ST}$  values between populations ranged from  $-0.008$  to  $0.032$  and were in most cases not significant (Tab. V).

### 3.4. Chloroplast microsatellites

A subset of two out of four analyzed cpSSR loci, namely Pt71936 and Pt87268, was polymorphic and used for further analyses.

### 3.5. Genetic variation within populations

For chloroplast microsatellites, 60 different haplotypes were observed. A total of 47 haplotypes and 50 haplotypes were observed in natural populations and plantations, respectively. A summary of chloroplast haplotype variation analyzed at two cpSSR loci is given in Table III. The average effective number of haplotypes was 14.34 for natural populations and 15.65 for plantations. Haplotypic diversity was very high in all populations. In natural populations it ranged from 0.942 (Bhaktapur NR) to 0.969 (Dadeldhura NR) with a mean haplotypic diversity of 0.953. In plantations it ranged from 0.922 (Parbat PL) to 0.983 (Dadeldhura PL) with a mean haplotypic diversity of 0.955.

### 3.6. Genetic differentiation among populations

Genetic distance ranged from 0.22 between Parbat NR and Bhaktapur PL to 1.19 between Palpa NR and Bhaktapur NR. The UPGMA dendrogram based on genetic distances between populations showed no association with geographic origin

**Table III.** Summary of chloroplast haplotype variation at two cpSSR loci.

|            | <i>N</i> | <i>Na</i> | <i>Ne</i> | <i>h</i> |
|------------|----------|-----------|-----------|----------|
| Natural    |          |           |           |          |
| Dadeldhura | 40       | 22        | 18.18     | 0.969    |
| Salyan     | 37       | 21        | 12.56     | 0.946    |
| Palpa      | 39       | 24        | 15.36     | 0.960    |
| Parbat     | 40       | 19        | 13.56     | 0.950    |
| Bhaktapur  | 38       | 17        | 12.03     | 0.942    |
| Mean       | 38.8     | 20.6      | 14.34     | 0.953    |
| Plantation |          |           |           |          |
| Dadeldhura | 40       | 28        | 24.24     | 0.983    |
| Salyan     | 38       | 22        | 16.79     | 0.966    |
| Palpa      | 39       | 21        | 13.00     | 0.947    |
| Parbat     | 37       | 17        | 9.71      | 0.922    |
| Bhaktapur  | 39       | 22        | 14.49     | 0.955    |
| Mean       | 38.6     | 22.0      | 15.65     | 0.955    |

*N*: sample size; *Na*: number of haplotypes; *Ne*: effective number of haplotypes; *h*: haplotype diversity.

**Table IV.** Summary of the non-hierarchical analysis of molecular variance (AMOVA) at cpSSRs.

| Source of variation             | <i>df</i> | Sums of square | Variation component | % variation | <i>p</i> value |
|---------------------------------|-----------|----------------|---------------------|-------------|----------------|
| Among groups                    | 1         | 2.97           | 0.006               | 0.35        | 0.1476         |
| Among populations within groups | 8         | 15.14          | 0.008               | 0.48        | 0.14           |
| Within populations              | 377       | 601.43         | 1.595               | 99.18       | 0.06           |

*df*: degree of freedom; *p* value: the probability of obtaining a more extreme component estimate by chance.

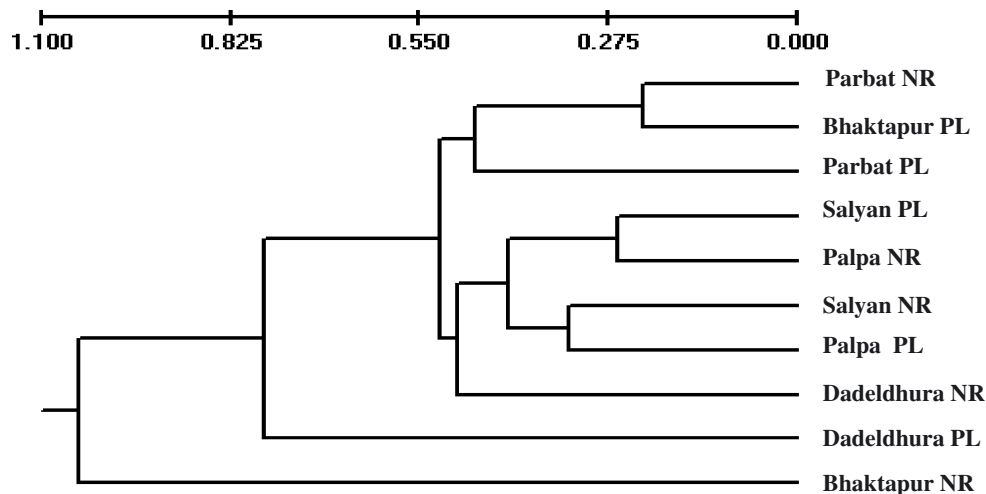
(Fig. 3). Planted and natural populations from the same region are found in different clusters of the dendrogram. For all clusters there is only low (non-significant) bootstrap support (< 50). Mantel tests showed no significant correlation between geographical and genetic distances for both natural populations ( $r = 0.3761$ ,  $P = 0.8052$ ) and plantations ( $r = 0.5626$ ,  $P = 0.100$ ).

Analysis of molecular variance (Tab. IV) showed a high proportion of variation within populations. Less than 1% of the total genetic variation occurred among populations demonstrating a very low population differentiation ( $F_{ST} = 0.008$ ). Also a very low differentiation of only 0.35% was found among the two groups (natural populations and plantations).

## 4. DISCUSSION AND CONCLUSION

### 4.1. Genetic variation within populations

We found moderate levels of genetic diversity within populations of *P. roxburghii* in Nepal at nuclear SSRs and high diversity of chloroplast haplotypes. Previous reports on the diversity of the species were based on isozymes (Hussain, 1995; Sharma et al., 2002) and used material from other regions of



**Figure 3.** UPGMA dendrogram based on Nei (1978) genetic distance between all studied natural populations and plantations (cpSSRs). Bootstrap values based on 10000 permutations (only bootstrap values above 50) are indicated in each node.

**Table V.** Pairwise  $F_{ST}$  and significant  $p$  values for the investigated ten populations (nSSRs).

| Dadeldhura NR | Dadeldhura PL | Salyan NR | Salyan PL | Palpa NR | Palpa PL | Parbat NR | Parbat PL | Bhaktapur NR | Bhaktapur PL |               |
|---------------|---------------|-----------|-----------|----------|----------|-----------|-----------|--------------|--------------|---------------|
| 0.000         | NS            | NS        | NS        | NS       | NS       | NS        | NS        | NS           | NS           | Dadeldhura NR |
| 0.023         | 0.000         | NS        | *         | NS       | NS       | NS        | NS        | NS           | NS           | Dadeldhura PL |
| 0.003         | 0.027         | 0.000     | NS        | NS       | NS       | NS        | NS        | *            | NS           | Salyan NR     |
| −0.001        | 0.028         | 0.001     | 0.000     | NS       | NS       | NS        | NS        | NS           | NS           | Salyan PL     |
| 0.002         | 0.021         | 0.006     | 0.008     | 0.000    | NS       | NS        | NS        | NS           | NS           | Palpa NR      |
| 0.003         | 0.010         | 0.011     | 0.018     | −0.008   | 0.000    | NS        | NS        | NS           | NS           | Palpa PL      |
| 0.012         | 0.021         | 0.018     | 0.022     | −0.003   | −0.004   | 0.000     | NS        | NS           | NS           | Parbat NR     |
| 0.002         | 0.032         | 0.012     | 0.011     | 0.009    | 0.012    | 0.030     | 0.000     | NS           | NS           | Parbat PL     |
| 0.006         | 0.013         | 0.011     | 0.020     | −0.002   | −0.005   | 0.004     | 0.009     | 0.000        | NS           | Bhaktapur NR  |
| −0.007        | 0.022         | 0.004     | 0.004     | 0.001    | 0.007    | 0.017     | 0.002     | 0.001        | 0.000        | Bhaktapur PL  |

NS: non significant \* significant at 5%.

the natural distribution of the species. These studies revealed high diversity within populations at isozyme gene loci. A direct comparison of results based on isozyme and nuclear SSR gene markers is not meaningful even though both marker types show co-dominant inheritance due to the typically higher diversity observed at nuclear SSRs.

The assessment of genetic variation within populations at nSSRs often revealed higher (e.g. *P. pinaster*: Mariette et al., 2001; *P. contorta*: Thomas et al., 1999; *P. canariensis*: Navascus and Emerson, 2007) or comparable (e.g. *Pinus strobus*: Marquardt et al., 2007) estimates to other pines. We investigated only three SSR loci, which were among other criteria selected due to their comparatively high polymorphism observed in a preliminary survey using few genotypes only. On the other hand, the investigated loci were developed for *Pinus taeda* and transferred to *P. roxburghii*, and LOP1 was developed from EST sequences. The polymorphism level is often lowered if a SSR marker is transferred to another species in comparison to the species used for SSR development (Weising et al., 2005) and selective constraints possibly restrict variation at SSRs in coding regions developed from EST sequences (Rungis et al., 2004).

While the conclusion of ‘moderate’ levels of genetic diversity in the natural populations of *P. roxburghii* in Nepal at nuclear genes is preliminary due to the low number of investigated SSRs and the considerations mentioned above, the large number of cpDNA haplotypes clearly indicates high diversity of the chloroplast genome, even though only two highly polymorphic cpSSRs were investigated. On average more than 20 haplotypes were identified in samples with a mean size of less than 40 trees per population. Because of their uniparental inheritance the effective population size of chloroplast genomes is half of nuclear diploid genomes.

Thus, cpDNA markers are expected to show stronger negative effects of reduced population sizes on diversity levels within populations in comparison to biparentally inherited nuclear markers (McCauley, 1995; Navascués and Emerson, 2007). Our results indicate that the genetic structures of the natural populations were not strongly affected by bottlenecks and genetic drift in their recent evolutionary past.

One of the main objectives of this study was the comparison of variation levels in natural and planted populations. Absolute levels of diversity are less important in this context. The average number of alleles per population was slightly lower in

plantations (4.93) than in natural populations (5.0) at nSSRs while the average observed heterozygosity was identical (0.50) in both population types. The average number of cpDNA haplotypes is even higher in plantations than in natural populations in contrast to results by Navascués and Emerson (2007) who investigated natural and artificially regenerated stands with unknown origin of *P. canariensis*. Thus, we reject hypothesis (ii) according to which the variation levels in plantations are lower than in natural populations. Neither the mode of seed harvest nor selective effects prior to or after the establishment of plantations caused noticeable losses of diversity at the observed nuclear gene markers or chloroplast haplotypes (see below). No significant impact of the regeneration method (natural regeneration versus plantation) on levels of genetic diversity at nSSRs was also reported by Thomas et al. (1999) for *P. contorta*.

Inbreeding coefficients (fixation indices  $F$ ) were close to 0 and sometimes even negative within both plantations and natural populations. However, comparatively high fixation indices were observed in the population pair Palpa (mean  $F = 0.17$  for the natural population and mean  $F = 0.18$  for the plantation). The observation of positive and rather homogeneous fixation indices at the three investigated nSSRs in the natural population Palpa suggests that inbreeding due to elevated levels of selfing or biparental mating is a possible cause of the positive  $F$ -values (Tab. I). A negative fixation index was observed at locus PtTX2123 in the plantation Palpa raising doubts that inbreeding caused the high fixation indices at the remaining two loci in this population. Other causes such as the occurrence of mutations at the primer binding sites next to one or more SSRs causing 'null alleles' with higher frequencies in the Palpa populations than in other stands cannot be ruled out.

## 4.2. Genetic variation among populations

Approximately 99% of the total variation resided within populations both at nSSRs and at cpDNA haplotypes. Accordingly, differentiation among populations is extraordinary low.

Low differentiation among natural populations is frequently observed at neutral marker loci for conifers and other wind-pollinated trees forming large, more or less continuous stands (Hamrick et al., 1992). Gene flow mainly through pollen and longevity are the main evolutionary factors responsible for the lack of strong differentiation among wind-pollinated trees (Austerlitz et al., 2000). Thus, extensive pollen-mediated gene flow is likely to be the main cause for the absence of significant differentiation among natural populations. Since no significant spatial structure was observed among natural populations, hypothesis (iv) is rejected. Efficient movement of pollen causes not only low differentiation among populations, but also prevents significant correlations between spatial and genetic structures at neutral markers in natural populations of *P. roxburghii* in Nepal.

Differentiation was also very low between natural populations and plantations. Genetic structures of plantations are very similar to those of natural populations, and genetic distances between natural populations and neighbouring plantations are

as low as those between natural stands and more distant plantations. Thus, we also reject hypothesis (i), since the two population types natural populations and plantations are not significantly differentiated from each other, and hypothesis (iii) since genetic distances between neighbouring plantations and natural populations are small. The origin of a stand (natural or planted) has no main impact on its genetic structures at the investigated loci.

## 4.3. Genetic consequences of plantation establishment

The establishment of the plantations had no noticeable effects on the genetic structures of the established man-made forests in comparison to natural stands. In particular, genetic diversity was not or only slightly reduced at nuclear microsatellites and chloroplast haplotypes. This result is mainly due to the low differentiation observed among natural stands as potential origins, and due to the mode of harvesting and production of seeds and seedlings for plantation establishment.

Natural stands are main sources for forest reproductive material in the absence of an advanced tree improvement programme for a plantation species in a particular region. The very low differentiation observed at the investigated gene markers makes it impossible to reliably identify the actual seed production areas used for seed harvesting operations in Nepal. However, our results do not support the idea that long-distance transport of forest reproductive material was involved in the establishment of the plantations. Even though the origin of the plantations cannot be identified, import of seeds from widely separated areas not covered in this survey or even other countries seems unlikely.

The mode of seed harvest and in particular the number of seed trees is of crucial importance for the genetic structure of the resulting plantations. The high diversity observed in all plantations and the low differentiation among the investigated populations strongly suggest that a high effective number of seed trees contributed to the planted progenies in all plantations, since a low number of seed trees inevitably results in decreased diversity and stronger differentiation among populations due to drift effects.

Mixing of material from several source populations potentially increases the diversity of plantations (Stefenon et al., 2008). However, the increase of diversity depends on the differentiation among source populations and disappears if populations are not differentiated from each other. Mixing of seeds from several poorly differentiated populations cannot be ruled out and may potentially contribute to the presumably large effective number of mother trees used for the production of seeds.

The similarity of genetic structures in planted and natural populations is in striking contrast to a study on genetic variation patterns of another important forest tree species in Nepal, *Dalbergia sissoo*. Pandey et al. (2004) also investigated five population pairs of *D. sissoo* consisting of a natural population and a neighbouring plantation in Nepal. They found almost complete differentiation between the groups of natural



and planted populations at maternally inherited cpDNA haplotypes. In addition, polymorphism was observed at a single isozyme gene locus (*Gdh-A*) in all natural populations, but all plantations were fixed on a single allele. Pandey et al. (2004) interpreted their results as a strong hint towards the importance of long-distance transfer of forest reproductive material for the establishment of *D. sissoo* plantations in Nepal. Material was possibly imported from India. The susceptibility of *D. sissoo* plantations in Nepal to diseases is likely to be related to the use of reproductive material which was transported over long distances and hence might be poorly adapted to the environmental conditions in Nepal.

The exact origin of plantations is unknown for both species and seed harvesting was not monitored. Strong differences between genetic structures of natural versus planted stands were observed for *D. sissoo*. The adaptive potential of plantations is likely to be negatively affected for this species. However, no comparable effects of plantation establishment on genetic structures were observed for *P. roxburghii*.

Uncontrolled production of seeds and seedlings is a main problem for plantation forestry not only in Nepal, but also in most tropical countries. The origin of most tree plantations in the tropics is unknown, which often raises concerns about the adaptive potential of man-made forests (Finkeldey and Hattermer, 2007). The comparison of the results described here to the study by Pandey et al. (2004) shows that uncontrolled seed harvest, production of seeds, and marketing of reproductive material has very different consequences for the genetic structures of the established plantations. It is neither justified to assume a 'narrow genetic base' for all plantations of tropical trees with unknown origin, nor is it wise to neglect the potential consequences of uncontrolled seed harvest for the adaptedness and adaptability of man-made forests (Finkeldey and Hattermer, 2007).

#### 4.4. Conservation of genetic resources

*P. roxburghii* is the most important forest tree species in Nepal. It is frequently planted and accordingly not in danger of immediate extinction in the country. However, genetic resources are possibly lost due to human impact such as fragmentation of natural forests and unsustainable resin tapping. In addition, large-scale afforestation and reforestation is expected to irreversibly modify genetic structures not only of newly established plantations, but also of surrounding remnants of natural forests by gene flow mainly through pollen from planted stands (Ellstrand, 1992).

Our results indicate that risks arising from plantation establishment for the genetic resources of *P. roxburghii* in Nepal are possibly low. Variation levels and genetic structures of all investigated plantations and natural forests are similar. This suggests that even plantations might qualify as gene conservation stands.

These conclusions are based on a small number of presumably selectively neutral gene markers, and refer to a set of five population pairs only. Similar genetic structures, in particular low differentiation among planted and natural popu-

lations, are also expected at other neutral gene loci and in most other populations growing under comparable conditions in Nepal. However, stronger differentiation may be expected at loci under selection due to different environmental conditions. The conservation of the variation at these currently unknown "adaptive" gene loci is of particular concern. Thus, we propose to establish at least four areas for the in situ conservation of genetic resources in Nepal, one in each of the main growing regions of the species in the country, to capture also possibly existing 'hidden' variation at adaptive gene loci.

As shown above, a large number of genetically highly variable *P. roxburghii* plantations exist in Nepal. The phenotypically best plantations should be developed into seed production areas as a first step towards an operational tree improvement programme and as ex situ conservation stands. We recommend to assess the diversity of selected in situ and ex situ conservation stands at marker loci or to select natural or planted populations included in this study in order to safely avoid a focus of costly conservation measures on populations with low diversity.

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